A putative binding protein for lipophilic substances related to butterfly oviposition

Kazuko Tsuchihara[a,b], Kohei Ueno[c], Akira Yamanaka[d], Kunio Isono[c], Katsuhiko Endo[d], Ritsuo Nishida[e], Kazuo Yoshihara[a], Fumio Tokunaga[b,*]

[a] *Suntory Institute for Bioorganic Research, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan*
[b] *Department of Biological Science and Department of Earth and Space Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*
[c] *Graduate School of Information Science, Tohoku University, Sendai, Miyagi 980-8577, Japan*
[d] *Department of Physics, Biology and Informatics, Faculty of Science, Yamaguchi University, Yamaguchi 753-8512, Japan*
[e] *Laboratory Chemical Ecology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract A unique protein of 23 kDa (Jf23) was found in the tarsus of the female swallowtail butterfly, *Atrophaneura alcinous*. Jf23 has 38% identity with a bilin-binding protein, which was found in the cabbage butterfly, *Pieris brassicae*, and which has two consensus sequences in common with the members of the lipocalin family, suggesting that it is a binding protein for lipophilic ligands. Western blot analysis showed that Jf23 was expressed only in the female, and not in the male. Electrophysiological response of the female tarsi was stimulated by methanolic extract of their host plant, Dutchman's pipe (*Aristolochia debilis*). The stimulated response was depressed by the presence of Jf23 antiserum. These results suggest that Jf23 is one of the chemosensory signaling proteins, which plays one or more roles in female butterfly oviposition.

Key words: Butterfly oviposition; Binding proteins for lipophilic substance; Chemosensory reception

1. Introduction

Sensory reception plays a very basic role in an animal's interaction with its environment. Photoreception and auditory reception have been studied extensively, but chemoreception, including taste and olfactory, have been studied less due to the difficulty in isolating the chemoreceptors involved. Nevertheless, olfactory signal transduction mechanisms have recently been studied at the gene and protein levels. Several odorant-binding proteins [1] and odorant receptors have been found in vertebrates [2]. Pheromone-binding proteins (PBPs) [3,4] and general odorant-binding proteins (GOBPs) have been found in the antennae of moths [5–7]. Also, PBP-related proteins (PBPRPs) have been isolated from *Drosophila melanogaster* [8], and lipophilic stimulant carrier proteins (CRLBPpr) from the blowfly [9]. All cysteine residues are preserved at the same positions in all the binding proteins described above (except those in vertebrates), suggesting that they belong to the same gene family.

It is well known that butterflies lay eggs on their host plants, for example, *Pieris brassicae* on Cruciferae, swallowtail butterflies, *Papilio xuthus*, on Rutaceae and *Atrophaneura alcinous* on Dutchman's pipe (Aristolochiaceae). The oviposition behavior of butterflies is induced by the recognition of the plant components at the fifth tarsi of their forelegs [10–12]. Actually, *A. alcinous* females have a toothbrush-like dense cluster of sensilla there, which is larger than that of the male [10–12]. Although chemical studies of the stimulants in the host plants have been reported for several species of butterflies, the chemoreception mechanism of butterflies for oviposition is not clear.

This work aims to clarify the chemotransduction mechanism of oviposition at the molecular level. Here we describe the isolation of a specific protein from female butterflies and its amino acid sequence, which has some identity to proteins of the lipocalin family. Also, we suggest that the protein plays some role in the sensory process for oviposition.

2. Materials and methods

2.1. Materials

Swallowtail butterflies were collected around Kizu-river Kyoto, Japan. Eggs were obtained by hand pairing, and larvae were fed with the host plant, Dutchman's pipe, *A. debilis*. The plant was collected from around Kizu-river and Setsunan University, Osaka, Japan. Adult butterflies were reared at 25°C and fed with 15% sucrose solution. Butterflies approximately 1 week old were used for the experiments. The leaves and stems of *A. debilis* were extracted with methanol to prepare the stimulating solution for the electrophysiological experiments.

2.2. Electrophoresis

The tarsi were isolated from 50 male and 50 female swallowtail butterflies, homogenized in liquid nitrogen, dissolved in 5 mM sodium barbiturate–HCl buffer (pH 6.8) by sonication, and then centrifuged at 3000 rpm for 20 min. The supernatant, after centrifugation, was concentrated using a 10 000 NMWL filter (Millipore Ultrafree) and subjected to SDS–PAGE. SDS–PAGE was performed on a gradient slab acrylamide gel (15–25% gradient) at 30 mA for 2 h. Proteins were stained with Coomassie brilliant blue. Size markers for SDS–PAGE were purchased from Pharmacia LMW.

2.3. Amino acid sequencing

The protein, which is specific to the female tarsi, was separated by SDS–PAGE and blotted onto the PVDF membrane filter (Millipore). The filter with the protein was subjected to an automatic Edman degradation using a protein sequencer (Shimadzu protein sequencer PPSQ-10).

2.4. Molecular cloning

The tarsi of 100 each of *A. alcinous* males and females were iso-

*Corresponding author. Fax: (81)-6-6850 5480.
E-mail: tokunaga@ess.sci.osaka-u.ac.jp

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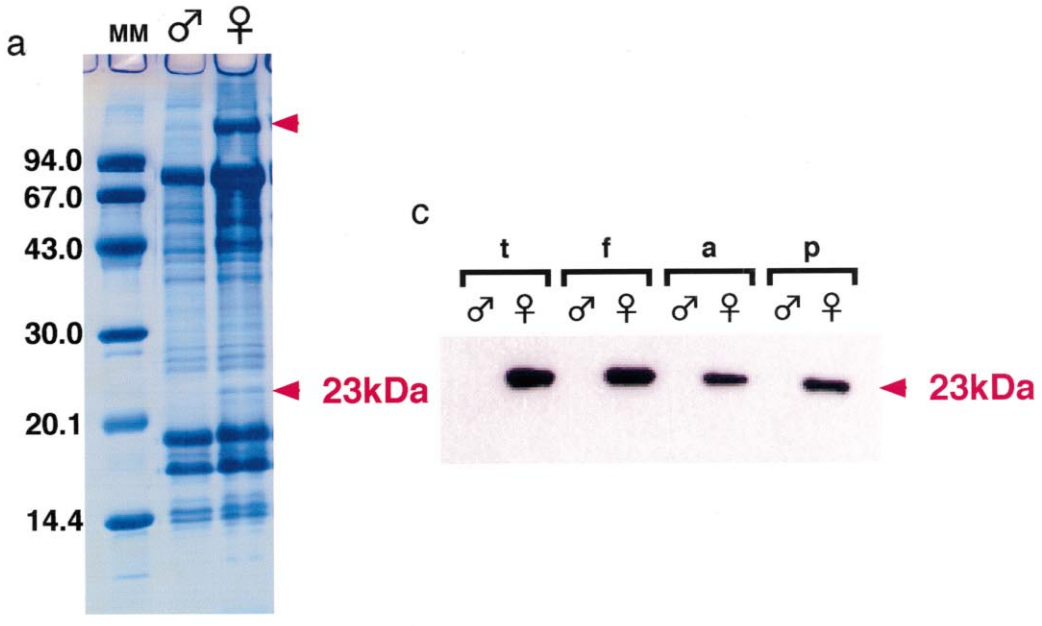

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                                    M  L  L  Y  F  L  A  A

CTGCTCGTGGTGGCGACGGCGGAGGTTTACAAAGATGGACCCTGCCCGGACGTGAGCCCC     120
L  L  V  V  A  T  A  E  V  Y  K  D  G  P  C  P  D  V  S  P      13

ATGGCGAACTTTGATCTTAATTCTTTTCAAGGAGCTTGGTACGAAATCGCTAGATACCCG     180
M  A  N  F  D  L  N  S  F  Q  G  A  W  Y  E  I  A  R  Y  P      33

AACGCCGAGGAAGAAGGCCAAAGGGCGAAGTGCTCGATTACTGAATACAACTTGAATGGA     240
N  A  E  E  E  G  Q  S  A  K  C  S  I  T  E  Y  N  L  N  G      53

AACAAGAATAAAGGCAAACTGATGTTCTCGCAAGTGGTGGATGGTGTAAGGTCATTTATT     300
N  K  N  K  G  K  L  M  F  S  Q  V  V  D  G  V  S  S  F  I      73

AGAGGCGACGTTTCACACGACGGACCTGCTAGATTAGTTTTCACTTATAACTTTGGGGGA     360
S  G  D  V  S  H  D  G  P  A  S  L  V  F  T  Y  N  F  G  G      93

TTGACGCGCGGTAATGAAATGACTATTCTGGACACCGACTACAAAAAATACGCCATCGGC     420
L  T  R  G  N  E  M  T  I  L  D  T  D  Y  K  K  Y  A  I  G     113

TACAGGTGCAAGGAGTTGAAGGATTCCAACAAACACCAAGTGTTCGCCTGGACCTTCTCC     480
Y  S  C  K  E  L  K  D  S  N  K  H  Q  V  F  A  W  T  F  S     133

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T  V  N  S  V  I  T  H  F  L  K  F  *  185

GGAAAATGAGCAAAGTTTTATAGAGATAAATTAAATAGCTTGAATATCAAAAAAAAAAAAA     720
AAA  723
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Fig. 1. Primary structure and expression of Jf23. a: Electrophoretic detection of Jf23. MM, ♂ and ♀ indicate the lanes of molecular markers, and male and female samples, respectively. Extracted sample prepared from tarsi of forelegs of 50 male and female butterflies. Arrow heads represent female specific bands. The 23 kDa band is Jf23. b: Nucleic acid and deduced amino acid sequences of Jf23. Molecular cloning of the Jf23 cDNA and deduced amino acid sequence. The nucleic acid and amino acid numbers are given on the right side of each line. The putative polyadenylation signal is underlined. The red line is the N-terminal amino acid sequencing yield. The two blue boxes are lipocalin family consensus sequences. c: Western blot analysis with antiserum against Jf23. t: tarsi, f: forelegs without tarsi, a: antenna, p: proboscis. ♂ and ♀ indicate the samples prepared from male and female, respectively.

lated, and then homogenized in liquid nitrogen. The homogenates were suspended in 2 ml of Trizol reagent (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. 50 μg of total RNA were extracted from the tissues. Using the superscript Preamplification System for 1st-strand cDNA synthesis (Gibco BRL), 20 ml of the 1st strand cDNA was prepared. The degenerative primers, FW primer (GA(A/G) GTI TA(C/T) AA(A/G) GA(C/T) GGI CC) and RV primer (GT(A/G)TG(A/G)TTIA(A/G)(A/G) TC(A/G) AA(A/G) TTI

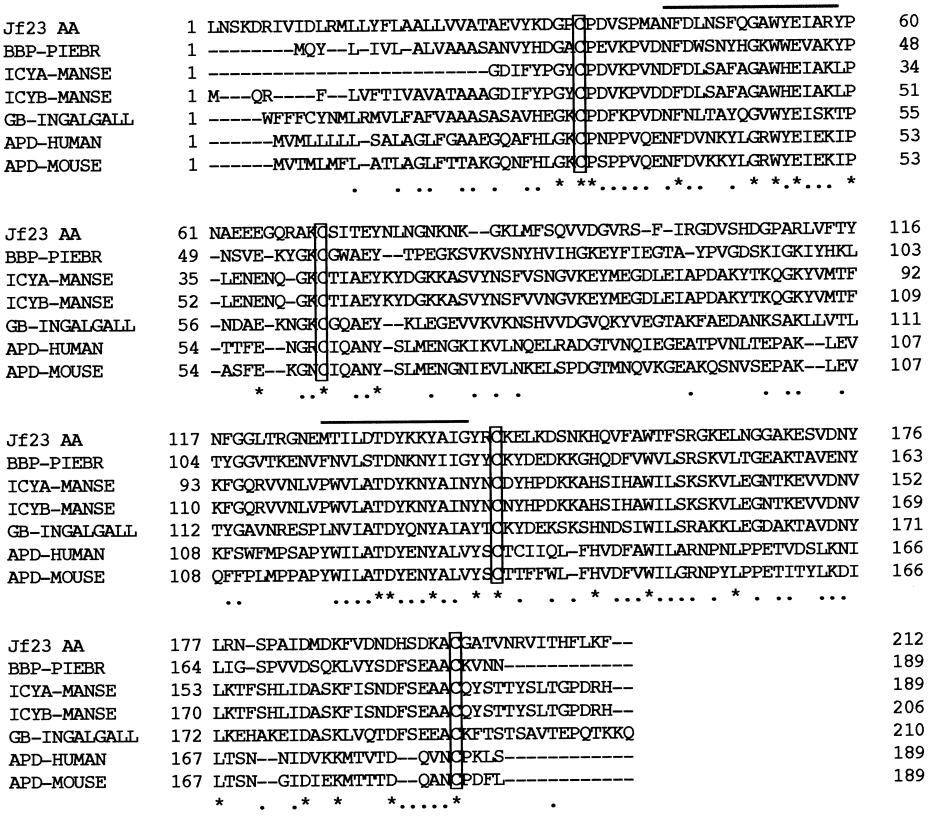

Fig. 2. Alignments of the amino acid sequence of Jf23 and the binding protein of BBP and lipocalin family. Jf23 AA: Jf23 protein, BBP–PIEBR: bilin-binding protein precursor, ICYA–MANSE: insectiamin A form precursor, ICYB–MANSE: insectiamin B form precursor, GB–INGALGALL: *Galleria mellonella* gallerin mRNA, complete cds, APD–HUMAN: Apolipoprotein D precursor (human), APD–MOUSE: Apolipoprotein D precursor (mouse). Amino acids are indicated with the single-letter code. The two lines are lipocalin family consensus sequences. The boxes indicate the position of the conserved cysteine residues.

GCC), corresponded to both sides of the determined amino acid sequence. Amplification was carried out with the primers using Ex *Taq* polymerase (Takara Shuzo, Kyoto, Japan) under the following conditions: 94°C for 5 min; 3 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and then 72°C for 5 min.

The amplified products were cloned into the pCR2.1 vector (Invitrogen, Groningen, The Netherlands) and their nucleotide sequences were determined using the ABI PRISM 310 (Perkin-Elmer) automated sequencing system with universal M13 primers. The 60 bp fragment was reacted at the 5′ end, internally and at the 3′ end. This 60 bp cDNA was then used with the 5′/3′-rapid amplification of cDNA (RACE) method (Boehringer Mannheim). Thus, the cDNA sequence was consistently obtained.

Using GENETYXMAC software, this putative amino acid sequence was analyzed for hydropathy and alignments of the amino acid sequence. The hydropathy plots were constructed using the method of Kyte and Doolittle [13].

2.5. Preparation of antiserum

An oligopeptide (EVYKDGPCPDVS-OH) was prepared on an automatic peptide synthesizer (ABI 431A peptide synthesizer), and was conjugated with bovine thyroglobulin (Sigma Chemical Co, St. Louis, MO, USA) with a heterobifunctional crosslinker, 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Boehringer Mannheim). The conjugated antigen was injected into two rabbits and an antiserum against the peptide was collected after three injections.

2.6. Western blot analysis

Proteins from the tarsi, forelegs, antennae and proboscis of male and female butterflies were separated by SDS–PAGE, and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with the antiserum against Jf23, diluted 1:3000. Specific antigens were detected by horseradish peroxidase conjugated anti-rabbit Fab (Boehringer Mannheim), diluted 1:3000, and detected by ECL-plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7. Electrophysiological experiments

There are approximately 60 female-specific chemosensilla on the first segment of the tarsus of the female foreleg. A tarsus was isolated from the foreleg of a mated female butterfly and used for electrophysiological recording. A reference glass electrode containing Ringer's solution (7.5 mg/ml of KCl and 0.35 mg/ml of NaCl) was inserted into the tarsus from the proximal cut end. Under the microscope, the tip of the chemosensillum on the 5th tarsal segment was stimulated with solutions in a glass capillary tube.

The stimulus solutions were prepared by maximally dissolving a methanolic extract of Dutchman's pipe (*A. debilis*) in a control solution containing 5% dimethyl sulfoxide (DMSO) and 50 mM choline chloride. Under the microscope, the tip of the chemosensillum was stimulated by a glass capillary with the solution in it. The capillary tube also served as a recording electrode [14]. For antiserum pretreatment, the sensillum was dipped in an antiserum solution diluted 1:500 in the control solution in a glass capillary tube for 10 min before stimulation.

The glass capillary tube for stimulation and the pretreatment capillary tubes were connected to a syringe by a tube so that the solution slowly flowed out from the tip by means of positive pressure to prevent changes in the concentration. Signals were amplified through a preamplifier (MEZ 7101:Nihon Kohden), filtered, digitized by an A/D converter and finally stored and processed on a computer (Lab PC+, National Instruments, Tokyo, Japan).

3. Results

3.1. Female butterfly specific protein, Jf23

The sensilla were isolated from the tarsi of male and female

A. alcinous. SDS–PAGE analysis of the tarsal proteins showed that two proteinic bands were found only in the female tarsi (Fig. 1a). The molecular masses of the two bands were ca. 100 and ca. 23 kDa. Since the molecular weights of many odorant-binding proteins reported so far are about 20 kDa [5–8], the 23 kDa protein, named Jf23, was blotted onto a PVDF membrane filter and its amino acid sequence was determined. N-terminal amino acid sequencing yielded the first 23 amino acids of the protein: Glu-Val-Tyr-Lys-Asp-Gly-Pro-Ser-Pro-Asp-Val-Ser-Pro-Met-Ala-Asn-Phe-Asp-Leu-Asn-Ser-Phe-Glu-. Since cysteine and serine can not be distinguished by Edman degradation, the correct sequence of this peptide was obtained by deduction from the nucleotide sequence of the cDNA of this protein.

Using degenerate primers designed on the basis of the first 20 amino acids, a 723 bp cDNA was finally amplified by PCR and completely sequenced. This putative protein encoded by the cDNA consists of 203 amino acid residues with a MW of 20 296 Da (Fig. 1b). The N-terminal amino acid sequence of Jf23 was determined using the amino acid sequence deduced from the nucleotide sequence of the cDNA. The protein contains a signal peptide of 15 amino acids. Jf23 contains 43% hydrophobic amino acid residues and the results also showed that it possesses four hydrophobic clusters of the amino acid sequence (data not shown).

3.2. Immunological experiments

The specificity of the antiserum is very high for Jf23. A 1:3000 dilution of the antiserum detected Jf23 in Western blot analysis (Fig. 1c). In order to investigate the localization of Jf23, various organs were investigated. The antibodies recognized only a single protein in the female tarsi, forelegs, antenna, and proboscis. All of the stained bands were located at 23 kDa. Jf23 is specifically expressed not only in the tarsi but also in other organs.

3.3. Comparison of Jf23 with other binding proteins

A comparison of the amino acid sequences of Jf23 with other binding proteins (Fig. 2) revealed a 38% identity of Jf23 sequences with those of the bilin binding protein of *P. brassicae* [15,16], and 30% identity with those of Apolipoprotein D (human and mouse) [17,18], gallerin [19], and insectiamins A and B [20]. The alignment of the four cysteine residues was preserved in all seven proteins (Fig. 2). Moreover, Jf23 has two consensus sequences in common with the lipocalin family and the typical G-X-W motif (Fig. 2). Jf23 (protein) and OBPs share consensus sequences (lipocalin family consensus) but the overall sequence similarity is low [1,3–9]. Therefore we think they belong to different families. Therefore Jf23 could belong to a lipophilic ligand-binding protein family.

3.4. Electrophysiological experiments

Electrophysiological responses were recorded from the sensilla of the female tarsus. Sensilla at the bottom of the fifth tarsus were chosen because of their high sensitivity. Stimulation of the sensilla with a methanolic extract of *A. debilis* evoked a train of impulses with multiple amplitudes (Fig. 3a). Two to three different trains of impulses with differently sized amplitudes were usually recognized. The control solution containing 5% DMSO and 50 mM choline chloride, on the other hand, did not evoke any response (Fig. 3b). Therefore, the sensilla contain two or three neurons responsive to the leaf extracts of *A. debilis*. The sensilla were then pretreated with the antiserum raised against Jf23 for 10 min and then stimulated with the leaf extracts. The response was partially suppressed by the pretreatment, as is shown in Fig. 3c. Note that the suppression of the response is more evident at the beginning of the measurements. The suppression was not observed when the tips were pretreated with normal serum (nonspecific serum) (Fig. 3d). When the sensillum was washed with water for 5 min after the antibody treatment, the responses to the leaf extracts of *A. debilis* recovered (Fig. 3e).

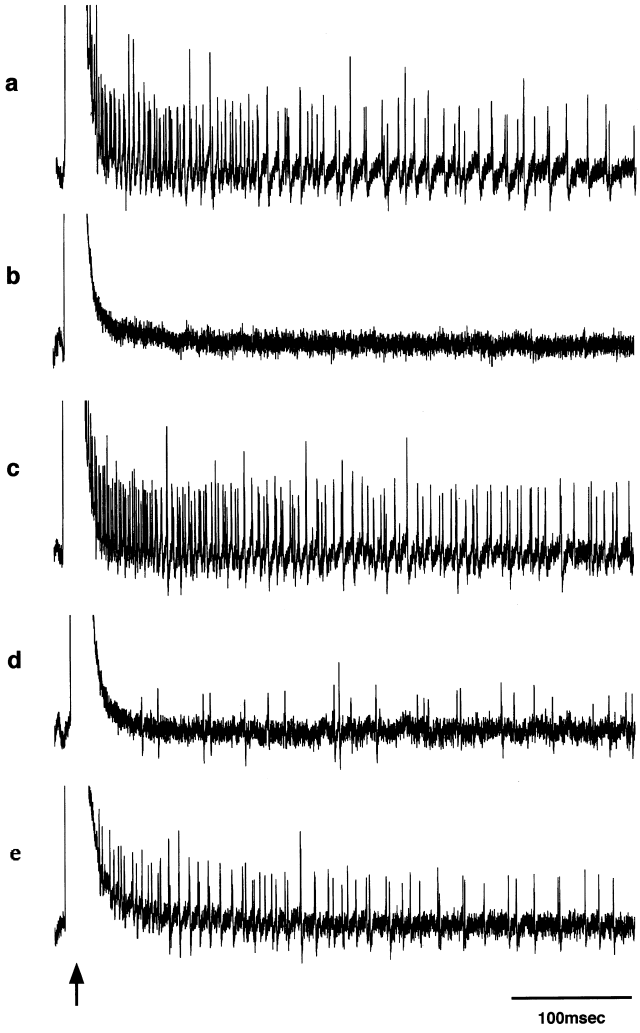

Fig. 3. Typical responses of the tarsal chemosensory sensilla a sesillia of female butterfly to host plant (*A. debilis*) extracts. a: Response to the methanolic extracts of *A. debilis*. b: Response to the control solution. c: Response to the methanolic extracts after 10 min treatment with antiserum against Jf23, (d) after 10 min treatment with normal serum, (e) the extracts after 5 min of washing with water. An arrow indicates the beginning of the stimulation. All recordings were obtained from a single identified sensilla.

4. Discussion

Oviposition behavior of butterflies is stimulated by chemicals, which are present in their host plants. Although oviposition of *P. brassicae* is evoked only by a single component, sinigrin (a thiocyanate glucosides), for *P. xuthus*, a combination of ten compounds are necessary. Some of them are lip-

ophilic. *A. alcinous* requires two compounds, hydrophilic sequoitol and lipophilic aristrochic acids. Actually, aristrochic acids are not sparingly soluble in the buffer solution used for the electrophysiological measurements, so it is necessary for aristrochic acids to contain DMSO for the measurements. Receptors are separated from the surface of the sensilla. Body fluids fill the space between them. A transportation system for lipophilic compounds should be required in the fluid.

Odorant-binding proteins such as PBPs, GOBPs, and PBPRPs are found in the chemosensory organs of both vertebrates and invertebrates. They have been characterized as binding proteins of lipophilic ligands. In the case of chemoreception in the chemosensilla of the butterfly for stimulating oviposition, the existence of such a protein was assumed [21]. Molecular cloning experiments using DNA sequences of these binding proteins, however, could not reveal any such protein (data not shown). Since oviposition behavior only occurs in females, and chemosensilla mainly appear on the fifth tarsus of the female, the 23 kDa protein, Jf23, which was only isolated on the SDS–PAGE gel from the female protein fraction, was tested with our target molecule.

Because Jf23 exhibits a rather high homology with the bilin-binding protein and lipocalin family proteins, all of which are binding proteins of lipophilic ligands, it was assumed that Jf23 is also a binding protein of a lipophilic substance. In addition to the SDS–PAGE of the protein fraction from the tarsi, the Western blotting experiments showed expression of Jf23 only in the female.

In the electrophysiological experiments with the antiserum, the response of the receptor neurons sensitive to the host plant extract was partially but significantly suppressed. The decreased response of the sensilla to the host plant extract due to pretreatment of the sensilla with the anti-Jf23 antiserum suggests that Jf23 is involved in the activation of the signaling process of the receptor. The suppression by anti-Jf23 antibody was removed by washing with water, suggesting that the binding of the antibody is reversible. These results strongly suggest that Jf23 is involved in the activation of the receptor molecules of the chemosensory neurons. Jf23 may carry and transfer the ligands involved in the leaf extracts to the receptor molecules or may activate the receptor molecules when it is bound to the ligand.

The results of the Western blot analysis and electrophysiological experiments, together with the identity of the amino acid sequence of Jf23 with other lipocalins, suggest that Jf23 plays the role of a binding receptor in the chemosensory signal transduction system for oviposition probably as carrier protein of the stimulants.

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References

[1] Dear, T.N., Campbell, K. and Rabbitts, T.H. (1991) Biochemistry 30, 10376–10382.
[2] Buck, L. and Axel, R. (1991) Cell 65, 175–187.
[3] Vogt, R.G. and Riddiford, L.M. (1981) Nature 293, 161–163.
[4] Van den Berg, M. and Ziegelberger, G. (1991) J. Insect Physiol. 37, 79–85.
[5] Vogt, R.G., Rybczynski, R. and Lerner, M.R. (1991) J. Neurosci. 11, 2972–2984.
[6] Maibeche-Coisne, M., Longhi, S., Jacquin-Joly, E., Brunel, C., Egloff, M.-P., Gastinel, L., Cambillau, C., Tegoni, M. and Nagnan-Le Meillour, P. (1998) Eur. J. Biochem. 258, 768–774.
[7] Vogt, R.G., Prestwich, G.D. and Lerner, M.R. (1991) J. Neurosci. 22, 74–84.
[8] Pikielny, C.W., Hasan, G., Rouyer, F. and Rosbash, M. (1994) Neuron 12, 35–49.
[9] Ozaki, M., Morisaki, K., Idei, W., Ozaki, K. and Tokunaga, F. (1995) Eur. J. Biochem. 230, 298–308.
[10] Nishida, R. (1977) Botyu-Kagaku 42, 33–140.
[11] Nishida, R. and Fukami, H. (1989) J. Chem. Ecol. 15, 2565–2575.
[12] Ichinose, T. and Honda, H. (1978) Appl. Entomol. Zool. 13, 103–114.
[13] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[14] Hodgson, E.S., Lettvin, J.Y. and Roeder, K.D. (1955) Science 122, 417–418.
[15] Schmidt, F.S. and Skerra, A. (1994) Eur. J. Biochem. 219, 855–863.
[16] Suter, F., Kayser, H. and Zuber, H. (1988) Biol. Chem. Hoppe-Seyler 369, 497–505.
[17] Bishop, R.E., Penfold, S.S., Holtje, J.-V. and Weiner, J.H. (1995) J. Biol. Chem. 270, 23097–23103.
[18] Seguin, D., Desforges, M. and Rassart, E. (1995) Mol. Brain Res. 30, 242–250.
[19] Filippov, V.A., Filippov, M.A., and Sehnal, F. (1995) Accession L41641, unpublished (from BLAST search).
[20] Li, W. and Riddiford, L.M. (1992) Eur. J. Biochem. 205, 491–499.
[21] Nishida, R. (1995) Their ecology and evolution biology, chapter 3, pp. 17–26.